Screening Assays

The present invention provides a method for modulating regulatory RNA-ligand interactions, e.g. for the identification, selection and validation of opener and closer nucleic acids for the controlled manipulation of RNA secondary structures. In a preferred embodiment the openers or closers of the present invention are used to modulate RNA secondary structure dependent RNA-ligand interactions and the associated downstream processes such as gene expression.

The present invention for example relates to the field of RNA biology, in particular to RNA secondary structure analysis and prediction and to RNA secondary structure dependent RNA-ligand interactions. The present invention is particularly related to RNA interactions with proteins involved in gene regulation, more specifically in the post transcriptional regulation of gene expression, particularly the control of mRNA stability, e.g. the mRNA stabilization by binding of the protein HuR (ELAVL1) to AU-rich element containing mRNAs.
The present invention relates for example further to the field of drug discovery, more specifically to the identification and validation of pharmaceutical targets, to the development of assays for high throughput screening and to the profiling of compounds. The present invention is for example further related to the manipulation of disease relevant RNA-ligand interactions, particularly to RNA-ligand interactions which are dependent on RNA secondary structure features, e.g. the manipulation of RNA-ligand interactions controlling the messenger RNA stability of disease relevant genes, e.g. the interactions between the protein HuR and AU-rich element mRNAs like early response gene mRNAs, e.g. the mRNAs of TNFα or IL-2. The present invention includes the development of nucleic acid tools for the manipulation of gene expression, which is related to other but complementary approaches such as antisense techniques or RNAi.

Many RNA associated regulatory processes in the cell critically depend on the formation of a specific RNA secondary structure. In the understanding of this invention the secondary structure ψ of an RNA molecule of sequence s is a list of base pairs (i.e. pairs of nucleotides of the sequence) satisfying the following conditions: (*i*) Each nucleotide s_i participates in at most one base pair; (*ii*) each base pair (s_i,s_j) of ψ is one of the canonical base pairs GC, CG, AU, UA, GU and UG; (*iii*) base pairs satisfy the non-pseudoknot condition, i.e. they do not cross: two base pairs (s_i,s_j) and (s_k,s_l) of ψ with $i < j$, $k < l$, and $i < k$ implies either $j < k$ or $j > l$.

Like other macromolecules, RNA molecules of equal sequence *s* may form many different structures, denoted by the set Σ(s). For each secondary structure ψ in Σ(s) one can compute a free energy F(ψ) (relative to a random coil structure) by adding up energy contributions for stacked base pairs, hairpin loops, interior loops, bulges and multi-branched loops. These contributions have been determined experimentally (Mathews D.H. et al., J.Mol.Biol.1999, 288(5):911-40). Structures are not evenly distributed in the thermodynamic equilibrium ensemble of structures but the frequency of a structure depends on its stability (i.e. its free energy) and can be computed as:

$$p(\Psi) = \frac{1}{Z}\exp(-\frac{F(\Psi)}{RT}) \qquad [I]$$

where $Z = \sum_{\gamma \in \Sigma(s)} \exp(-F(\gamma)/RT)$ is the partition function of the RNA molecule *s*, *T* is the temperature and *R* is the universal gas constant.

As a consequence of the foregoing, a secondary structure motif or secondary structure element, required for a particular biological process may not be formed by all structures in the thermodynamic equilibrium ensemble of a sequence but only by a subset $A(s) \subseteq \Sigma(s)$. Structures forming a secondary structure motif or element required for a particular biological purpose are herein designated as active or accessible structures throughout the present invention. If a particular secondary structure motif can be formed only once within a secondary structure, the probability of accessible structures $p_\cdot$ in the thermodynamic ensemble can be computed by summing over the probabilities of individual structures in *A*(*s*):

$$p_\cdot = \sum_{\Psi \in A(s)} p(\Psi) = \frac{1}{Z}\sum_{\Psi \in A(s)} \exp\left(-\frac{F(\Psi)}{RT}\right) = \frac{Z_\cdot}{Z} \qquad [II]$$

where $Z_\cdot$ denotes a constrained partition function, i.e. a partition function of an ensemble of structures constrained to accessible structures.

The computation of $p_\cdot$ is more complicated if the secondary structure motif of interest may be formed several times within one structure. In this case, $p_\cdot$ is redefined to the probability of structures which form at least one required motif. The probabilities that the motif is formed at position B_i and that the motif is formed at position B_j within the same structure are not independent. Therefore, sums over individual probabilities have to be corrected for joint occurrences. For *M* sites B_i, *i*=1..*M*, in sequence *s* where the motif can be formed, $p_\cdot$ can be

calculated using the exclusion-inclusion principle (see e.g. Meisner N.C. et al., Chembiochem 2004, 5(10):1432-47; Hackermüller J. et al., Gene, 2005, in press).

$1 - p_\bullet = \sum_{\ell=0}^{M} (-1)^\ell \sum_{\substack{a \\ \lvert a\rvert=\ell}} p(a)$	[III]

where $p(a)$ is the probability of structures that form the motif at a subset of sites $a \subseteq \{B_1, B_2, \ldots, B_M\}$, which can be calculated again as a fraction of partition functions $p(a) = Z(a)/Z$. $Z(a = \varnothing)$ means that there is no constraint on the ensemble (and hence $p(\varnothing) = 1$), while $a = \{B_1, B_2, \ldots, B_M\}$ means that the motif is formed at all possible sites. The thermodynamics of an RNA molecule *M* may change dramatically when it hybridizes with a short oligonucleotide *O*. Consequently, hybridization of an oligonucleotide may change the tendency of RNA molecules to form a particular secondary structure motif. More precisely, the probability of structures in the thermodynamic ensemble that form at least one structure motif of interest may be different in a structure ensemble of RNA-oligonucleotide hybrids as compared to the structure ensemble of the RNA molecule alone. The overall probability $p_\bullet$ of secondary structures forming at least one motif of interest depends on the probability of accessible structures of the RNA molecule $p_\bullet^M$, the probability of accessible structures of the RNA-oligo hybrid $p_\bullet^{MO}$ and on the equilibrium between hybridized and unhybridized RNA, i.e. on the equilibrium concentrations of oligonucleotide [*O*] and RNA [*M*] and the hybridization energy between RNA and oligonucleotide expressed by the equilibrium constant of the hybridization, K_{MO}. Under the assumptions that (*i*) the oligonucleotide is nearly complementary to the RNA; (*ii*) oligonucleotide and RNA are not significantly self complimentary and (*iii*) the oligonucleotide is present in excess, one may approximate $p_\bullet$ by $p_\bullet^{MO}$ (Hackermueller J. et al., Gene, 2005, in press).

Specific RNA – protein interactions are of crucial importance in the regulation of numerous cellular mechanisms. Pre mRNA processing, nuclear (m)RNA export, RNA stability and degradation, other regulatory levels of gene expression, metabolic processes and viral lifecycle regulation critically depend on the specific recognition of RNA molecules by protein factors. For many of these processes it has been shown that either RNA secondary structure motifs augment or enable the recognition of (degenerate) sequence motifs or that "pure"

RNA secondary structure motifs without sequence constraints are recognized by regulatory proteins: It has been shown recently that the apparent affinity of an RNA protein interaction which requires a particular RNA secondary structure motif depends directly on the probability of accessible (i.e. motif forming) secondary structures in the thermodynamic equilibrium ensemble of the RNA sequence

$K_d^{app} = K_d \frac{1}{p_\bullet}$	[IV]

where $K_d^{app} = [R][P]/[RP]$ is the apparent dissociation constant and $K_d = [R_\bullet][P]/[RP]$ is the microscopic dissociation constant describing the affinity between protein and accessible RNA molecules. [*R*], [*R*•], [*P*], [*RP*] denote the respective equilibrium concentrations of RNA, accessible RNA, protein and the RNA protein complex.

A dependence on RNA secondary structure has recently been shown for the interaction between the protein HuR and AU-rich element mRNAs. AU-rich elements (ARE) are *cis* acting elements predominately present in the 3' untranslated regions (UTR) of early response gene mRNAs. These loosely defined elements are essentially characterized by a combination of AUUUA and UUAUUUA(U/A)(U/A) motifs and U-rich regions. Their presence targets a messenger RNA for rapid degradation *in cis*, and their function is dependent on the specific interaction with trans-acting factors. Among the at least 21 ARE-binding proteins identified so far, a positive regulatory effect has been attributed only to the ubiquitously expressed HuR (ELAVL1, RefSeq accession: NP_001410) and its neuronal homologues HuB, HuC, HuD. The interaction between HuR and an ARE containing mRNA leads to a stabilization of the bound mRNA and may determine the expression of approximately 3,000 genes (see e.g. Bakheet T. et al., Nucleic Acid Res. 2003, 60(3):499-511, including a number of disease relevant proteins and pharmaceutical targets in cancer, inflammatory, viral, allergic, vascular and infectious diseases. The list of currently known HuR controlled genes is given in Meisner N.C. et al., Chembiochem 2004, 5(10):1432-47 and encompasses BMP6 (bone morphogenetic protein 6), CCL11 (chemokine (C-C motif) ligand 11), eotaxin, CSF2 (colony stimulating factor 2), GMCSF (granulocyte monocyte colony stimulating factor), FSHB (follicle stimulating hormone beta), IL1b (interleukin 1 beta), IL2 (interleukin 2), IL3 (interleukin 3), IL4 (interleukin 4), IL6 (interleukin 6), IL8 (interleukin 8), MYOD1 (myogenic factor 3), MYOG (myogenin), NF1 (neurofibromin 1), PITX2 (paired-like homeodomain transcription factor 2), TNFα (tumor necrosis factor alpha), VEGF (vascular endothelial growth factor), CCNA2 (cyclin A), CCNB1 (cyclin B1), CCND1 (cyclin D1),

CCND2 (cyclin D2), CD83, CDKN1A (cyclin-dependent kinase inhibitor 1A, i.e. p21 or Cip1), CDKN1B (cyclin-dependent kinase inhibitor 1B, i.e. p27 or kip1), DEK, FOS (v-fos FBJ murine osteosarcoma viral oncogene homolog, c-fos), HLF (hepatic leukemia factor), JUN (v-jun sarcoma virus 17 oncogene homolog (avian), c-jun), MYC (v-myc myelocytomatosis viral oncogene homolog, c-myc), MYCN (v-myc myelocytomatosis viral related oncogene, neuroblastoma derived, n-myc), TP53 (tumor protein p53), HDAC2 (histone deacetylase 2), MMP9 (matrix metalloproteinase 9), NDUFB6 (NADH dehydrogenase (ubiquinone) 1 beta subcomplex), NOS2A (nitric oxide synthase 2A), PLAU (urokinase plasminogen activator), PTGS2 (prostaglandin-endoperoxide synthase 2), COX2 (cyclooxygenase 2), SERPINB2 (serine (or cysteine) proteinase inhibitor), PAI-2 (plasminogen activator inhibitor 2), UBE2N (ubiquitin-conjugating enzyme E2N), ADRB1 (beta-1-adrenergic receptor), ADRB2 (beta-2 adrenergic receptor), AR (androgen receptor), CALCR (calcitonin receptor), CDH2 (cadherin 2, type 1), N-cadherin, GAP43 (growth associated protein 43), SLC2A1 (solute carrier family 2 member 1), GLUT1 (glucose transporter 1), PLAUR (urokinase plasminogen activator receptor), SLC5A1 (solute carrier family 5), TNFSF5 (tumor necrosis factor (ligand) superfamily, member 5, CD154), ACTG1 (actin, gamma 1), CTNNB1 (catenin (cadherin-associated protein), beta 1), MARCKS (myristoylated alanine-rich protein kinase C substrate), MTA1 (metastasis associated 1), PITX2 (paired-like homeodomain transcription factor 2), SLC7A1 (cationic amino acid transporter, CAT-1). Binding of the positive regulator HuR to an ARE mRNA is determined by the presence of its binding site NNUUNNUUU in single stranded conformation.

The present invention combines the foresaid aspects, i.e. the ability to influence RNA thermodynamics by hybridization of short oligonucleotides and the dependence of RNA protein interactions on the formation of a particular secondary structure element. This invention provides methods to rationally design short oligonucleotides which manipulate the probability of secondary structures that form a particular secondary structure element required for a particular biological process, e.g. a secondary structure element required for an RNA-ligand interaction.

RNA secondary structure is of central importance in the regulation of many cellular processes such as translation, mRNA processing, metabolic pathways or other control mechanisms depending on RNA-ligand interactions. The present invention provides a method for controlled manipulation of RNA secondary structure and the associated

regulatory processes. Computationally designed oligonucleotides hybridize to their target RNA molecule and thereby maximize or minimize the probability of a particular secondary structure element which is crucial for the molecular interaction of interest. These modulator (*opener* or *closer*) nucleic acids not only allow to e.g. unfold a regulatory hairpin but to disrupt or favor any functional RNA structure element. Such an artificially induced conformational reorganization may occur at regions proximal or distant to the hybridization site within the target RNA sequence. It allows to hide or present the recognition site of a regulatory factor and may thereby be used to manipulate the associated regulatory process. In particular we describe its application for specifically controlling the expression of disease relevant target genes at the level of HuR dependent mRNA stabilization. Given the disease relevance of many HuR regulated genes, targeting mRNA stability by manipulation of mRNA secondary structure by a method of the present invention could serve as a novel platform strategy for therapeutic intervention. The method therefore provides a means for *(i)* the development of specific target related assays and HTscreens to identify compounds which inhibit or strengthen the modulator induced structure change on a disease relevant target mRNA, (*ii*) for boosting or silencing gene expression as a tool in cell biological or *in vivo* assays, (*iii*) in drug target validation or *(iv)* in compound profiling, and *(v)* for mechanistic studies of the biological role of one particular protein binding site by specifically "opening" or "closing" an individual among multiple binding sites.

In one aspect the present invention provides a method for modulating regulatory RNA-ligand interactions comprising

(a) defining and selecting a secondary structure element of an RNA molecule which is required for the recognition by a ligand, e.g. protein,

(b) calculating the thermodynamic probability of the secondary structure element of step a) in the secondary structure ensemble of said RNA,

(c) calculating the thermodynamic probability of the secondary structure element of step a) in the secondary structure ensemble of said RNA hybridized to an at least partly reverse complementary oligonucleotide,

(d) determining an oligonucleotide that changes the thermodynamic probability of said secondary structure element beyond a defined probability threshold,

(e) providing an oligonucleotide as determined in step (d), and optionally,

(f) hybridizing an RNA comprising said secondary structure element of step (a) to an oligonucleotide of step (e), and determining the effect of said hybridization on the thermodynamic probability of said secondary structure element.

If not defined otherwise herein the terms are defined as follows:
An *RNA* of the present invention includes any RNA molecule with a biological function, including covalently modified RNA, e.g. from the group consisting of messenger RNA (mRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA), transfer RNA (tRNA), micro RNA (miRNA), small nucleolar RNA and spliceosomal RNA. Preferably, RNA is an mRNA, such as an AU-rich element mRNA, e.g. from the group consisting of BMP6 (bone morphogenetic protein 6), CCL11(chemokine (C-C motif) ligand 11), eotaxin, CSF2 (colony stimulating factor 2), GMCSF (granulocyte monocyte colony stimulating factor), FSHB (follicle stimulating hormone beta), IL-1b (interleukin 1 beta), IL-2 (interleukin 2), IL-3 (interleukin 3), IL-4 (interleukin 4), IL-6 (interleukin 6), IL-8 (interleukin 8), MYOD1 (myogenic factor 3), MYOG (myogenin), NF1 (neurofibromin 1), PITX2 (paired-like homeodomain transcription factor 2), TNFα (tumor necrosis factor alpha), VEGF (vascular endothelial growth factor), CCNA2 (cyclin A), CCNB1 (cyclin B1), CCND1 (cyclin D1), CCND2 (cyclin D2), CD83, CDKN1A (cyclin-dependent kinase inhibitor 1A, i.e. p21 or Cip1), CDKN1B (cyclin-dependent kinase inhibitor 1B, i.e. p27 or kip1), DEK, FOS (v-fos FBJ murine osteosarcoma viral oncogene homolog, c-fos), HLF (hepatic leukemia factor), JUN (v-jun sarcoma virus 17 oncogene homolog (avian), c-jun), MYC (v-myc myelocytomatosis viral oncogene homolog, c-myc), MYCN (v-myc myelocytomatosis viral related oncogene, neuroblastoma derived, n-myc), TP53 (tumor protein p53), HDAC2 (histone deacetylase 2), MMP9 (matrix metalloproteinase 9), NDUFB6 (NADH dehydrogenase (ubiquinone) 1 beta subcomplex), NOS2A (nitric oxide synthase 2A), PLAU (urokinase plasminogen activator), PTGS2 (prostaglandin-endoperoxide synthase 2), COX2 (cyclooxygenase 2), SERPINB2 (serine (or cysteine) proteinase inhibitor), PAI-2 (plasminogen activator inhibitor 2), UBE2N (ubiquitin-conjugating enzyme E2N), ADRB1 (beta-1-adrenergic receptor), ADRB2 (beta-2 adrenergic receptor), AR (androgen receptor), CALCR (calcitonin receptor), CDH2 (cadherin 2, type 1), N-cadherin, GAP43 (growth associated protein 43), SLC2A1 (solute carrier family 2 member 1), GLUT1 (glucose transporter 1), PLAUR (urokinase plasminogen activator receptor), SLC5A1 (solute carrier family 5), TNFSF5 (tumor necrosis factor (ligand) superfamily, member 5, CD154), ACTG1 (actin, gamma 1), CTNNB1 (catenin (cadherin-associated protein), beta 1), MARCKS (myristoylated alanine-rich protein kinase C

substrate), MTA1 (metastasis associated 1), PITX2 (paired-like homeodomain transcription factor 2) and SLC7A1 (cationic amino acid transporter, CAT-1), e.g. the RNA is a mRNA, such as an IL-2 mRNA or a TNF-α mRNA.

A ligand of the present invention includes a molecule which binds to an RNA molecule, e.g. including an RNA, DNA, peptide, protein or a small molecule, e.g. a protein, such as an ARE binding protein, e.g. of the ELAV family, such as ELAVL1 (HuR), HuB, HuC, HuD, e.g. ELAVL1.

A *secondary structure element* or, synonymously used, a *secondary structure motif* of the present invention includes an RNA secondary structure pattern consisting of paired and unpaired positions, e.g. a hairpin, such as a hairpin of eight stacked bases closing a loop of 5 bases. Alternatively, a secondary structure element or secondary structure motif includes a combined sequence/RNA secondary structure motif, such as a sequence pattern with a secondary structure constraint, preferably, a sequence pattern with defined secondary structure, e.g. the sequence pattern NNUUNNUUU in fully single stranded conformation, where N is the IUPAC code for any RNA nucleotide. Preferably, the secondary structure element or secondary structure motif is required for a particular biological function, e.g. the binding of a particular protein to the RNA or the autocatalytic cleavage of the molecule, preferably, the secondary structure motif is required for the binding of HuR to an mRNA.

The probability of RNA secondary structures in the thermodynamic equilibrium ensemble of a particular sequence that form a particular secondary structure element required for a biological process, such as the probability of RNA secondary structures that contain the sequence motif NNUUNNUUU in fully single stranded conformation, is herein also designated as *accessibility*. The thermodynamic equilibrium ensemble may be of any RNA molecule, such as an mRNA, or of an RNA molecule hybridized with oligonucleotides, such as an mRNA hybridized with openers or closers as defined below.

An *oligonucleotide* of the present invention includes all nucleoside oligomers of defined sequence, preferably RNA, DNA, PNA (peptoid nucleic acids) or LNA (locked nucleic acids) with any 2'- or backbone modification such as 2'oxy-methyl or phosphorothioate-substitutions. An oligonucleotide of the present invention may also be present in a labeld form, e.g. labeled at the 3' or 5' terminus or at internal positions with labeling substances as

conventional, such as e.g. biotin, cholesterol, digoxigenin, colloids, transition element complexes, carbohydrates, amino acids, or with fluorescent, radioactive, alkyl-, peptide or other tags or with synthetic polymers, e.g. polylysine or (poly)ethyleneglycol.
The length of the oligonucleotide depends on the secondary structure of the RNA and the desired sequence specificity, preferably the oligonucleotide has a length of 10 to 200 nucleotides, such as 10 to 100, preferably 10 to 50, such as about 20 nucleotides.

The oligonucleotide of the present invention may act as an opener or as a closer.
An *opener* of the present invention includes an oligonucleotide which raises the accessibility of the hybrid of a particular RNA with said opener of a particular secondary structure element beyond a defined threshold compared to the accessibility of said RNA of said secondary structure element, e.g. a threshold defined as a value which is at least 2 times higher. An opener is partly reverse complementary to the RNA, preferably it is exactly reverse complementary.

A *closer* of the present invention includes an oligonucleotide which lowers the accessibility of the hybrid of a particular RNA with said closer of a particular secondary structure element beyond a defined threshold compared to the accessibility of said RNA of said secondary structure element, e.g. a threshold defined as a value which is at least 0.5 times lower. A closer is partly reverse complementary to the RNA, preferably it is exactly reverse complementary.

The present invention further provides a method for the identification and selection of oligonucleotides which act as openers or closers for a particular RNA and a particular secondary structure element. A prerequisite is the prior knowledge of the secondary structure element required for a particular biological process of interest, e.g. for binding of a ligand, e.g. a protein. The secondary structure element may e.g. be common knowledge or identified from the literature. For RNA-ligand interactions, a required secondary structure element can be identified, e.g. from affinity data, using a method as conventional, e.g. such as a method which is described in detail in Hackermueller J. et al., 2005, Gene (in press) or which is exemplified for the interaction between HuR and mRNAs as described in Meisner N.C. et al. Chembiochem, 2004, 5(10:1432-47.

The accessibility $p_{\bullet}^{M}$ is calculated for said RNA secondary structure element and said RNA, e.g. by using a computational method as given in Computational Protocols. Depending on the computational method it may be possible to set the temperature for which the accessibility is calculated. Preferably, the temperature is set to the ambient temperature at which the oligonucleotide is experimentally validated or applied, e.g. to 37° for *in vivo* use.

The accessibility $p_{\bullet}^{MO}$ of the RNA –oligonucleotide hybrid is calculated for candidate openers or closers. Candidate openers or closers may be arbitrarily chosen oligonucleotides which are at least partly reverse complementary to the RNA, or may be chosen as partly reverse complementary oligonucleotides which hybridize to the RNA in proximity of positions where said secondary structure element can be formed. Preferably, $p_{\bullet}^{MO}$ is evaluated for all oligonucleotides of a defined length, e.g. 20 nucleotides (nt) which are exactly reverse complementary to said RNA.

An oligonucleotide is selected as an opener if the difference between $p_{\bullet}^{M}$ and $p_{\bullet}^{MO}$ is beyond a certain threshold, e.g. $p_{\bullet}^{MO}$ is twice as high as $p_{\bullet}^{M}$. An oligonucleotide is selected as a closer if the difference between $p_{\bullet}^{M}$ and $p_{\bullet}^{MO}$ is beyond a certain threshold, e.g. $p_{\bullet}^{MO}$ is half as high as $p_{\bullet}^{M}$.

Optionally, such identified openers or closers may be further validated experimentally by hybridizing them with said RNA and determining the effect of the hybridization on the accessibility of the secondary structure element, e.g. by known methods such as ELISA (enzyme linked immunosorbant assay), immuneprecipitation, filter binding assays, EMSA (= electrophoretic mobility shift assay), UV/VIS spectroscopy, NMR (nuclear magnetic resonance spectroscopy), fluorescence spectroscopy with a particular focus on applications with single molecule sensitivity e.g. Fluorescence Correlation Spectroscopy (FCS), Fluorescence Intensity Distribution Analysis (FIDA), or applications based on the determination of Fluorescence Anisotropy or Fluorescence Resonance Energy Transfer (FRET).

In a preferred aspect the present invention provides a method of the present invention wherein the RNA is an IL-2 mRNA, the ligand is ELAVL1 and the oligonucleotide has a sequence selected from the group consisting of

SEQ ID NO 1: AAGGCCTGATATGTTTTAAG,
SEQ ID NO 2: AATATAAAATTTAAATATTT,
SEQ ID NO 3: TAGAGCCCCTAGGGCTTACA,
SEQ ID NO 4: TGAAACCATTTTAGAGCCCC,
SEQ ID NO 5: AAGGCCUGAUAUGUUUUAAG,
SEQ ID NO 6: AAUAUAAAAUUUAAAUAUUU,
SEQ ID NO 7: UAGAGCCCCUAGGGCUUACA,
SEQ ID NO 8: UGAAACCAUUUUAGAGCCCC.

In another preferred aspect the present invention provides a method of the present invention wherein the RNA is a TNF-α mRNA, the ligand is ELAVL1 and the oligonucleotide has a sequence selected from the group consisting of
SEQ ID NO 9: TCGGCCAGCTCCACGTCCCG,
SEQ ID NO 10: TCTGGTAGGAGACGGCGATG,
SEQ ID NO 11: ACGGCGATGCGGCTGATGGT,
SEQ ID NO 12: TTCTGGAGGCCCCAGTTTGA,
SEQ ID NO 13: ATTCCAGATGTCAGGGATCA, and
SEQ ID NO 14: ATCACAAGTGCAAACATAAA,

In another aspect the present invention provides the use of a method of the present invention for manipulating the expression of a gene by altering the secondary structure of the corresponding RNA.

In a further aspect the present invention provides an oligonucleotide that changes the thermodynamic probability of a secondary structure element beyond a defined probability threshold identified by a method of the present invention.

In another aspect the present invention provides an oligonucleotide having a sequence selected from the group consisting of
SEQ ID No 1: AAGGCCTGATATGTTTTAAG,
SEQ ID No 2: AATATAAAATTTAAATATTT,
SEQ ID No 3: TAGAGCCCCTAGGGCTTACA,
SEQ ID No 4: TGAAACCATTTTAGAGCCCC,
SEQ ID No 5: AAGGCCUGAUAUGUUUUAAG,

SEQ ID No 6: AAUAUAAAAUUUAAAUAUUU,
SEQ ID No 7: UAGAGCCCCUAGGGCUUACA,
SEQ ID No 8: UGAAACCAUUUUAGAGCCCC,
SEQ ID No 9: TCGGCCAGCTCCACGTCCCG,
SEQ ID No 10: TCTGGTAGGAGACGGCGATG;
SEQ ID No 11: ACGGCGATGCGGCTGATGGT;
SEQ ID No 12: TTCTGGAGGCCCCAGTTTGA,
SEQ ID No 13: ATTCCAGATGTCAGGGATCA, and
SEQ ID No 14: TCACAAGTGCAAACATAAA.

In a preferred aspect the oligonucleotide identified by a method of the present invention is an RNA or DNA molecule or an oligonucleotide of SEQ ID NO 1 to SEQ ID NO 14, with any chemical modification;
e.g. a modification selected from the group consisting of 2`-O-methyl-
Phosphorothioate-, Cholesterol-, Biotin- and fluorescence dye.

In another preferred aspect the oligonucleotide identified by a method of the present invention or an oligonucleotide of SEQ ID NO 1 to 14 is a PNA or LNA molecule.

An oligonucleotide identified by a method of the present invention or an oligonucleotide of SEQ ID NO 1 to SEQ ID NO 14 are hereinafter designated as "an oligonucleotide of the present invention".

In another aspect the present invention provides the use of an oligonucleotide identified by a method of the present invention or an oligonucleotide selected from the group consisting of SEQ ID No 1 – 14 for manipulating regulatory RNA-ligand interactions, e.g. as a tool in the manipulation of RNA secondary structure, e.g. to modulate RNA-ligand interactions such as RNA protein interactions, preferably mRNA protein interactions, and the regulatory effects associated with this interaction. In particular, oligonucleotides identified by a method of the present invention may be used for the manipulation of gene expression, e.g. the expression of AU-rich element controlled genes, preferably by manipulation of HuR controlled mRNA stability as illustrated in Figure 1.

The function of the opener or closer oligonucleotides provided by the present invention merely depends on the ability to specifically hybridize with its target RNA. Hence, they provide a great flexibility with respect to chemical modifications. This allows to adjust the physico- and biochemical properties of the opener or closer oligonucleotide. For example, single stranded RNA, DNA, PNA (peptoid nucleic acids) or LNA (locked nucleic acids) with any 2'- or backbone modification such as 2'oxy-methyl- or phosphorothioate- substitutions may be used. Also, the opener or closer oligonucleotides may be labeled at the 3' or 5' terminus or at internal positions with biotin, cholesterol, digoxigenin, colloids, transition element complexes, carbohydrates, amino acids, or with fluorescent, radioactive, alkyl-, peptide or other tags or with synthetic polymers, e.g. polylysine or (poly)ethyleneglycol. The opener or closer oligonucleotides may be used in *in vitro*, cellular or *in vivo* applications. For example, opener or closer oligonucleotides of the present invention may be delivered to cells by transfection methods such as adjuvant mediated transfer, e.g. transfer by liposome, DEAE dextran, calcium phosphate or other adjuvants, by viral vectors, e.g. retroviral vectors, or by physical methods, e.g. electroporation, microinjection or optoinjection. For *in vivo* assays, the openers or closers may be delivered e.g. orally, by injection, e.g. subcutaneously, intramuscularly or intravenously, by inhalation, rectally, topically or by a microprojectile approach ("Gene Gun").

For example, manipulation of gene expression by opener or closer oligonucleotides of the present invention may be applied for the validation of drug targets, e.g. by comparing a phenotypic readout for an opener increased expression of a disease relevant gene versus normal expression or a decreased expression of the same gene, mediated by a closer of the present invention or by conventional methods, e.g. RNAi, antisense or other knockdown methods. In another example, manipulation of gene expression by opener or closer oligonucleotides of the present invention may be used for mechanistic studies in *in vivo*, cell biological or biochemical assays, e.g. by comparing a phenotypic readout for an opener increased expression of a gene of interest versus normal expression or a decreased expression of the same gene, mediated by a closer of the present invention or by conventional methods, e.g. RNAi, antisense or other knockdown methods. Said phenotypic readout is e.g. selected from the group consisting of a change in RNA secondary structure, RNA tertiary structure, RNA-ligand complex levels, RNA-ligand affinities, RNA oligo- or multimerization, ligand oligo- or multimerization, autocatalytic RNA cleavage, ligand conformation, RNA splicing, covalent RNA modifications, RNA localization, RNA stability, RNA expression levels, protein expression levels, RNA or protein localization, cell

proliferation, cell differentiation, cell migration, inflammation, tissue vascularization, tumour progression, angiogenesis or any other downstream effect of the RNA secondary structure which is manipulated by an oligonucleotide of the present invention.

In another example, manipulation of gene expression by opener or closer oligonucleotides of the present invention may be applied for the profiling of agents, e.g. a chemical substance, by e.g. comparing the effect of said agent at an opener increased expression of a gene of interest versus normal expression or a decreased expression of the same gene, mediated by a closer of the present invention or by conventional methods, e.g. RNAi, antisense or other knockdown methods.

In another aspect the present invention provides the use of an oligonucleotide identified by a method of the present invention or an oligonucleotide selected from the group consisting of SEQ ID No 1 – SEQ ID NO 14 for influencing the stability of an RNA molecule.

In a further aspect the present invention provides:

(I) an assay for identifying an agent that modulates the effect of the hybridization of an RNA molecule to an oligonucleotide comprising

(a) hybridizing an RNA comprising a secondary structure element which is required for recognition by a ligand to an oligonucleotide that changes the thermodynamic probability of said secondary structure element beyond a defined probability threshold in the presence and in the absence of a candidate compound,

(b) determining the effect of hybridization of said RNA to said oligonucleotide in the presence and in the absence of said candidate compound, and

(c) identifying an agent which modulates the effect of hybridization.

(II)an assay for identifying an agent that mimics the effect of hybridization of an RNA molecule to an oligonucleotide comprising

(a)hybridizing an RNA comprising a secondary structure element which is required for recognition by a ligand to an oligonucleotide that changes the thermodynamic probability of said secondary structure element beyond a defined probability threshold,

(b)hybridizing an RNA comprising a secondary structure element which is required for recognition by a ligand to a candidate compound which is expected to have a similar effect as the oligonucleotide,

(c)determining the effect of hybridization for steps (a) and (b), and

(d)identifying an agent which mimics the effect of hybridization of step (a).

A candidate compound includes compound (libraries) from which hybridization according to the present invention may be expected, e.g. including RNA fragments, DNA fragments, oligopeptides, polypeptides, proteins, antibodies, mimetics, small molecules, e.g. low molecular weight compounds (LMW's), preferably LMW.

An agent is one of the chosen candidate compounds, for which a hybridizing effect as described above has been proven.
Agents like small molecules or other chemical substances may be tested for either inhibiting, enhancing or simulating this effect, e.g. by binding to a specific conformation in the mRNA structure during rearrangement.

In a preferred aspect the effect of hybridization in the assays of the present invention is determined by measuring a signal which is related to the effect of hybridization, which effect is selected from the group consisting of changes in secondary RNA structure, tertiary RNA structure, RNA-ligand affinity, RNA oligo- or multimerization, ligand oligo- or multimerization, conformational change of the ligand, efficiency of a downstream effect of RNA-ligand recognition, RNA splicing, covalent RNA modifications, RNA localization, RNA stability,RNA translation and protein expression profiles.

Such assays may be realized by hybridizing an oligonucleotide of the present invention to an RNA of interest in presence or absence of a candidate compound and measuring a phenotypic readout associated with the effect of said hybridization to identify an agent which modulates or mimics said effect. The effect of said hybridization is e.g. determined by measuring a signal which is related to a phenotypic readout as described above.
For example, said assays can be used for making ARE controlled genes drugable at the level of mRNA regulation. Assuming that the cell uses modulators like small RNAs or proteins to induce mRNA structure changes strongly related or similar to the openers or closers of the present invention, the openers or closers of the present invention may be used to design target specific mRNA stability assays. Exemplary assay principles using openers of the present invention are illustrated in Figures 11 and 12: The opener leads to a structural rearrangement, detected e.g. by standard fluorescence spectroscopic methods like fluorescence intensity, lifetime or fluorescence resonance energy transfer (FRET) measurements.

As outlined in Figure 13, the expansion to cellular assay formats is also possible. A prerequisite, however, is the efficient introduction of probe and/or opener oligonucleotides into the living cell. Having a prototype assay established, virtually any target mRNA within a target platform of ~ 3,000 ARE genes should become screenable using a common assay format.

In a preferred aspect the RNA in said assays is an mRNA.

In another preferred aspect the RNA, the ligand and the oligonucleotide are as defined above.

In another aspect the present invention provides the use of the assays of the present invention for high throughput screening.

In a further aspect, the present invention provides oligonucleotides or agents identified by a method of the present invention or an oligonucleotide selected from the group consisting of SEQ ID No 1 – SEQ ID NO 14 for use as a pharmaceutical.

In another aspect the present invention provides a pharmaceutical composition comprising an agent identified by an assay of the present invention or an oligonucleotide of the present invention beside at least one pharmaceutical excipient, e.g. appropriate carrier and/or diluent, e.g. including fillers, binders, disintegrators, flow conditioners, lubricants, sugars and sweeteners, fragrances, preservatives, stabilizers, wetting agents and/or emulsifiers, solubilizers, salts for regulating osmotic pressure and/or buffers.

In another aspect the present invention provides a pharmaceutical composition of the present invention, further comprising another pharmaceutically active agent.

Such compositions may be manufactured according, e.g. analogously to a method as conventional, e.g. by mixing, granulating, coating, dissolving or lyophilizing processes. Unit dosage forms may contain, for example, from about 0.5 mg to about 1000 mg, such as 1 mg to about 500 mg.

For use as a pharmaceutical, an agent or an oligonucleotide of the present invention includes one or more agents or oligonucleotides, e.g. a combination of agents or oligonucleotides.

The pharmaceutical compositions of the present invention may be used for the treatment of a disorder having an etiology associated with a downstream effect of the RNA secondary structure which is manipulated by said oligonucleotide or agent. For example, said downstream effect may be the production of a substance, e.g. a protein, e.g encoded by an AU-rich element controlled gene, preferably selected from the group consisting of cytokines, chemokines, growth factors, proto-oncogenes, viral proteins, receptors, hormones or enzymes, preferably such substance is selected from the group consisting of BMP6 (bone morphogenetic protein 6), CCL11(chemokine (C-C motif) ligand 11), eotaxin, CSF2 (colony stimulating factor 2), GMCSF (granulocyte monocyte colony stimulating factor), FSHB (follicle stimulating hormone beta), IL-1b (interleukin 1 beta), IL2 (interleukin 2), IL-3 (interleukin 3), IL-4 (interleukin 4), IL-6 (interleukin 6), IL-8 (interleukin 8), MYOD1 (myogenic factor 3), MYOG (myogenin), NF1 (neurofibromin 1), PITX2 (paired-like homeodomain transcription factor 2), TNFα (tumor necrosis factor alpha), VEGF (vascular endothelial growth factor), CCNA2 (cyclin A), CCNB1 (cyclin B1), CCND1 (cyclin D1), CCND2 (cyclin D2), CD83, CDKN1A (cyclin-dependent kinase inhibitor 1A, i.e. p21 or Cip1), CDKN1B (cyclin-dependent kinase inhibitor 1B, i.e. p27 or kip1), DEK, FOS (v-fos FBJ murine osteosarcoma viral oncogene homolog, c-fos), HLF (hepatic leukemia factor), JUN (v-jun sarcoma virus 17 oncogene homolog (avian), c-jun), MYC (v-myc myelocytomatosis viral oncogene homolog, c-myc), MYCN (v-myc myelocytomatosis viral related oncogene, neuroblastoma derived, n-myc), TP53 (tumor protein p53), HDAC2 (histone deacetylase 2), MMP9 (matrix metalloproteinase 9), NDUFB6 (NADH dehydrogenase (ubiquinone) 1 beta subcomplex), NOS2A (nitric oxide synthase 2A), PLAU (urokinase plasminogen activator), PTGS2 (prostaglandin-endoperoxide synthase 2), COX2 (cyclooxygenase 2), SERPINB2 (serine (or cysteine) proteinase inhibitor), PAI-2 (plasminogen activator inhibitor 2), UBE2N (ubiquitin-conjugating enzyme E2N), ADRB1 (beta-1-adrenergic receptor), ADRB2 (beta-2 adrenergic receptor), AR (androgen receptor), CALCR (calcitonin receptor), CDH2 (cadherin 2, type 1), N-cadherin, GAP43 (growth associated protein 43), SLC2A1 (solute carrier family 2 member 1), GLUT1 (glucose transporter 1), PLAUR (urokinase plasminogen activator receptor), SLC5A1 (solute carrier family 5), TNFSF5 (tumor necrosis factor (ligand) superfamily, member 5, CD154), ACTG1 (actin, gamma 1), CTNNB1 (catenin (cadherin-

associated protein), beta 1), MARCKS (myristoylated alanine-rich protein kinase C substrate), MTA1 (metastasis associated 1), PITX2 (paired-like homeodomain transcription factor 2) and SLC7A1 (cationic amino acid transporter, CAT-1).

Treatment includes treatment and prophylaxis.

For such treatment, the appropriate dosage will, of course, vary depending upon, for example, the chemical nature and the pharmakokinetic data of an agent or an oligonucleotide of the present invention employed, the individual host, the mode of administration and the nature and severity of the conditions being treated. However, in general, for satisfactory results in larger mammals, for example humans, an indicated daily dosage is in the range from about 0.01 g to about 1.0 g, of an agent or an oligonucleotide of the present invention; conveniently administered, for example, in divided doses up to four times a day.

An agent or an oligonucleotide of the present invention may be administered by any conventional route, for example enterally, e.g. including nasal, buccal, rectal, oral, administration; parenterally, e.g. including intravenous, intramuscular, subcutanous administration; or topically; e.g. including epicutaneous, intranasal, intratracheal administration;e.g. in form of coated or uncoated tablets, capsules, injectable solutions or suspensions, e.g. in the form of ampoules, vials, in the form of creams, gels, pastes, inhaler powder, foams, tinctures, lip sticks, drops, sprays, or in the form of suppositories.
An agent or an oligonucleotide of the present invention may be administered in the form of a pharmaceutically acceptable salt, e.g. an acid addition salt or metal salt; or in free form; optionally in the form of a solvate. An agent or an oligonucleotide of the present invention in the form of a salt exhibit the same order of activity as the compounds of the present invention in free form; optionally in the form of a solvate.

An agent or an oligonucleotide of the present invention may be used for pharmaceutical treatment according to the present invention alone or in combination with one or more other pharmaceutically active agents. Combinations include fixed combinations, in which two or more agents or oligonucleotides of the present invention are in the same formulation; kits, in which two or more agents or oligonucleotides of the present invention in separate formulations are sold in the same package, e.g. with instruction for co-administration; and

free combinations in which the agents or oligonucleotides of the present invention are packaged separately, but instruction for simultaneous or sequential administration are given.

Description of the Figures

Figure 1: Schematic illustration of the opener action

Without opener action, HuR binding is impaired by inaccessibility of the binding site (marked by 1). Upon hybridization of the opener (marked by 2) to the target mRNA (mRNA α), specific modulation of the local secondary structure within the α mRNA leads to a presentation of the HuR binding site in accessible conformation resulting in mRNA α stabilization without affecting any other HuR target mRNA (e.g. mRNA ß).

Figure 2: Design of IL-2 opener oligonucleotides

The HuR binding site accessibility p^*_{MO} (i.e. fraction of accessible RNA) at 37° is shown in dependence of IL-2 3'UTR hybridization to a reverse complementary 20mer oligonucleotide at a given hybridization site (start position, x-axis) within the 3'UTR. The IL-2 ARE is indicated as a white box, NNUUNNUUU HuR binding sites are shown as blocks in the continuous black line at opener start positions about 50 and about 150. A significant increase in the accessibility is restricted to discrete "hotspots" in proximity of the HuR binding sites. The location of experimentally tested opener molecules Op_1, Op_2 Op_3 and Op_4 is indicated, negative controls (N_1, N_2) are also marked (sequences specified in Table 1). Opener Op_1 and Op_3 target primarily the HuR binding site within the ARE, Op_2 and Op_4 are directed to the second NNUUNNUUU motif.

Figure 3: Illustration of opener hybridization to the IL-2 3'UTR

Minimum free energy (MFE) secondary structure of the IL-2 3'UTR (left panel) and of the IL-2 3'UTR hybridized to opener Op_1 (right panel, Op_1 represented as a dark full line). NNUUNNUUU elements are marked as small spots in the right panel. As illustrated in the MFE conformation of the complex, the opener shifts the equilibrium towards conformations with accessible (i.e. single stranded) NNUUNNUUU elements – following the model sketched in Figure 1. Importantly, the opener effect does not necessarily need to be reflected in MFE structures, as the ensemble of structures is relevant for opener prediction. MFE secondary structures for 3'UTR – opener complexes are computed using Cofold (available with the Vienna RNA package).

Figure 4: IL-2 openers increase *in vitro* HuR affinity to IL-2 3'UTR

The apparent affinity of recombinant HuR to IL-2 3'UTR is determined in presence and absence of the openers with 1D-FIDA detection. All 4 tested IL-2 specific openers enhance the HuR association with the IL-2 3'UTR, reflected by a decrease in the apparent dissociation constant K_d^{app} (with Op_1: K_d^{app} = 11.80 ± 1.48 nM; with Op_2: K_d^{app} = 18.91 ± 1.91 nM, with Op_3: K_d^{app} = 8.38 ± 1.18 nM, with Op_4: K_d^{app} = 19.52 +/- 2.20 nM; without opener: K_d^{app} = 32.77 ± 4.48 nM; IL-2 3'UTR at 0.5 nM; openers at 25, 25, 5 and 1 nM, respectively). Hybridization of the negative control oligonucleotides to the IL-2 3'UTR leaces the interaction with HuR unaffected (with N_1: K_d^{app} = 32.91 ± 6.34 nM; with N_2: K_d^{app} = 32.77 ± 3.72 nM, N_1 and N_2 at 25 nM concentration) (A). The affinity increase induced by opener hybridization shows a saturation curve with half maximal saturation at an opener concentration of 0.38 nM (apparent affinity of Op_3 hybridization = 134 (± 54) pM). The apparent affinity of HuR binding to the opened IL-2 3'UTR approaches a maximum at a K_d^{app} of 8.57 ± 1.33 nM, both at 1.56 nM; IL-2 3'UTR at 0.5 nM in all experiments) (B). In presence of Op_1, the dissociation constant decreases with increasing opener concentrations to a minimum of K_d^{app} = 11.80 ± 1.48 nM at an Op1 concentration of 1.56 nM (IL-2 3'UTR at 2.5 nM in all experiments). For Op_1, increasing the concentrations beyond this optimum reverts the effect and the dissociation constant increases again.

Figure 5: IL-2 mRNA openers increase endogenous HuR-IL2 mRNA association

HuR mRNA complexes are co-immunoprecipitated from lysates of human PBMC without or after treatment with opener or negative control oligonucleotides Op_1, Op_2, N_2 and Op_T. HuR-bound IL-2 mRNA is quantified by real time RT-PCR. IL-2 mRNA amounts are normalized to the levels in untreated cells (open bars). Openers are added to 2.5 µM (hedged bars) or 10 µM (solid bars), negative controls N_2 and Op_T to 10 µM concentration. Both openers boost HuR mRNA complexation to up to 6.5 fold (Op_1) or 3.1 (Op_2) fold higher levels.

Figure 6: IL-2 mRNA openers inhibit IL-2 mRNA degradation

Degradation of endogenous IL-2 mRNA is monitored in human PBMC lysates. Upon addition of Mg^{2+} (t = 0 minutes), the amount of remaining IL-2 mRNA is quantified over time in the presence and in the absence of openers Op_1, Op_2 or N_2 at (A) 10 µM, (B) 25 µM and (C) 40 µM concentration by quantitative real-time RT-PCR. All data represent averages from at

least 3 independent samples and are normalized to the levels at timepoint t = 0 minutes. The data are fitted to a single exponential decay (solid line: no opener; dashed line: N_2). IL-2 mRNA is rapidly degraded at a halflife of $t_{1/2}$ = 8.34 ± 0.96 minutes (A), $t_{1/2}$ = 8.44 ± 1.98 minutes (B), and $t_{1/2}$ = 8.84 ± 2.19 minutes (C) without any opener (open circles), as well as in presence of 10 µM negative control N_2 (crosses), (A), $t_{1/2}$ = 6.82 ± 1.96 min). Addition of openers Op_1 (filled circles) or Op_2 (filled triangles) promotes a transient IL-2 mRNA stabilization in a concentration dependent manner (openers at 10µM in (A), 25µM in (B) and 40 µM in (C)). At 40 µM concentration (C), Op_1 blocks the degradation over the entire incubation time of 70 minutes. Op_2 shows a similar stabilizing effect, although it targets another HuR binding site (filled triangles, (B) and (C)). EF-1α, a non-ARE mRNA, remains stable over the entire observation time of 70 minutes (B)).

Figure 7: IL-2 opener oligonucleotides specifically promote IL-2 mRNA stabilization
The specificity of the opener induced mRNA stabilization is tested by monitoring their effect on the decay of other ARE containing cytokine mRNAs (TNF-α (A) and IL-1β (B)). TNF-α and IL-1 β mRNA degradation are characterized by a halflife of $t_{1/2}$ = 36.0 ± 2.2 minutes (open circles, (A)) and $t_{1/2}$ = 37.6 ± 5.6 minutes (open circles, (B)), respectively. In presence of either of the IL-2 specific openers Op_1 (filled circles) or Op_2 (filled triangles, both at 25 µM), neither TNF-α nor IL-1 β mRNA decay are altered.

Figure 8: Design of TNF-α openers and closers
The HuR binding site accessibility p^*_{MO} (i.e. fraction of accessible RNA) at 37° is shown in dependence of TNF-α mRNA hybridization to a reverse complementary 20mer oligonucleotide at a given hybridization site (start position, x-axis) within the mRNA (RefSeq NM_000594). The TNF-α 3'UTR is depicted by a black line, the ARE is indicated as a white box, NNUUNNUUU HuR binding sites are shown as black small boxes. Hybridization sites with a significant change in the accessibility are clustered in "hotspots", mainly in proximity but also distant to the HuR binding sites. Remarkably, also putative "closer" positions are identified, predicted to significantly decrease the NNUUNNUUU accessibility. Interestingly, these positions are located within the opener hotspot region. The location of putative openers, closers and negative control oligos are also shown as small boxes, respectively. The sequences of the identified TNF-α openers/closers (Op_A, Op_B, Cl_C, Op_E, Op_H) including the experimentally tested Op_T, Cl_T and N_T are specified in Table 1.

Figure 9: TNF-α openers increase *in vitro* HuR affinity to TNF-α 3'UTR
The apparent affinity of recombinant HuR to TNF-α 3'UTR is determined in the presence and in the absence of the opener, closer or negative control oligonucleotides in a 1D-FIDA assay. The TNF-α closer Cl_T significantly reduces the HuR association with the TNF-α 3'UTR, reflected by an increase in the apparent dissociation constant K_d^{app}. The effect correlates with the concentration of the closer. The effect approaches a maximum at the highest closer concentration with a ~ 2.5 fold Kd increase (with Cl_T: K_d^{app} = 13.80 ± 2.41 nM, without closer: K_d^{app} = 5.63 ± 0.87 nM) (A). The opener Op_T increases the affinity of HuR to the TNF-α 3'UTR by a factor of up to ~ 2 (B), which is in good consistence with the computationally predicted effect (Figure 8). However, at opener concentrations above 0.5 nM, the effect revertes again towards increased dissociation constants. Hybridization with a negative control oligonucleotide N_T does not affect the HuR – TNF-α 3'UTR affinity over the entire concentration range of the experiment (C). The TNF-α 3'UTR concentration is 1 nM in all experiments.

Figure 10: TNF-α openers specifically promote TNF-α mRNA stabilization
An opener designed for TNF-α (Op_T, see Figure 8 and Table 1) specifically stabilizes the TNF--α mRNA (A) without affecting IL-1β mRNA levels (B). (circles: without opener; stars: with opener Op_T at 25 µM).

Figure 11: Opener based *in vitro* assay design I: conformational switch
An exemplary assay principle for the identification of compounds acting on the ARE mRNA conformation is schematized. The opener induced conformational rearrangement is e.g. detected by appropriately positioned fluorophores, constituting a FRET pair. Strategically placed within the mRNA, their distance and, hence, the FRET efficiency becomes diagnostic for open or closed HuR binding site (marked by ⊗). As sketched in (A), an opening of the HuR binding site induced by a computationally designed opener is detected by a decrease in the FRET efficiency. (B) Low molecular weight compounds might interfere with this rearrangement by different modes of action. By binding to the mRNA, they may either compete directly with opener hybridization or locally freeze the mRNA in the closed conformation. Such compounds, characterized by a persistent energy transfer in presence of the opener, will prevent HuR from binding to the mRNA and thereby down-regulate the corresponding target gene. On the other hand, the opener effect may be enhanced by a compound which stabilizes the opened conformation. These compounds as well as such

which mimic the opener induced conformational switch are identified by a reduced energy transfer in presence or absence of the opener, respectively. The main issue for an adaptation to the HTS format will be the preparation of site specifically double labeled mRNA in sufficient amounts. An alternative strategy is therefore outlined in Figure 12.

Figure 12: Opener based *in vitro* assay design II: HuR binding

Alternatively, the assay strategy may be shifted to the detection of the HuR binding event by measuring energy transfer between two fluorophores on the mRNA and on HuR. The mRNA label may be either (A) covalently attached to the 3' terminus (e.g. as described in Qin P.Z. et al., Methods, 1999, 18(1):60-70) or (B) introduced via the opener oligonucleotide. In this setup, the conformational rearrangement induced by the opener is measured indirectly by a determination of HuR mRNA association, which is the subsequent step. Therefore, HuR inhibitors will produce the same signal as compounds which act on the mRNA conformation and need to be sorted out in an appropriate counterscreen.

Figure 13: Strategies for mRNA opener based cellular assays

Exemplary strategies for cellular assay design on ARE mRNA conformational switches are outlined (A). The target mRNA is specifically visualized by an appropriately designed fluorescent probe (e.g. Cy5). The probe is computationally designed to ensure minimal influence on the thermodynamic mRNA ensemble and to be compatible with the opener induced structural rearrangement. Endogenous HuR is labeled by fusion to a fluorescent protein (e.g. CFP). In the closed mRNA conformation, the two fluorophores (HuR, mRNA) will be spatially discrete. Upon binding of HuR, induced by the opener, the two fluorescent molecules will co-localize on the mRNA, which can e.g.be detected by high resolution confocal imaging (B). Alternatively, the mRNA specific label is placed directly on the opener oligonucleotide. In this setup, only opened mRNA molecules are detected and the mRNA structural ensemble is not affected by an (additional) probe. Provided that the probe hybridizes in sufficient proximity to the HuR binding site (marked by ⊗), energy transfer (i.e. donor quenching/acceptor sensitization) is the preferable detection mode to discriminate closed from open (i.e. HuR bound) conformations. Analogously to the illustration in Figure 11, compounds which inhibit or enhance the opener induced effect can be identified based on the discrimination of open versus closed mRNA conformations. A counterscreen using a constitutively "open" mRNA might be appropriate to sort out HuR inhibitors.

Legends to the Tables:

Table 1: Identified opener/closer oligonucleotides

Sequences of putative opener or negative control oligoribonucleotides identified for IL-2 or TNF--α are specified. Openers/closers which are selected for experimental validation, as well as negative control oligos are depicted in bold. The sequences are reverse complementary to the specified region in the target mRNA and given in 5' to 3' direction.

Table 2. Primers used for RT PCR

The sequences of the primers used for RT-PCR quantification of the target mRNAs of IL-2, TNF-α and IL-1β as well as for EF-1-α as internal control mRNA are specified in 5' to 3' direction.

In the following examples all temperatures are given in degree Celsius (°) and are uncorrected.

The following ABBREVIATIONS are used:

ACN	acetobitrile
BSA	bovine serum albumin
dsDNA	double stranded DNA
EDTA	N,N,N',N'-ethylenediaminetetraacetic
EF-1α	Elongation factor-1α
FCS	fetal calf serum
FIDA	Fluorescence Intensity Distribution Analysis (1D-FIDA = one dimensional or 2D-FIDA = 2 dimensional FIDA)
FCS	Fluorescence correlation spectroscopy
hPBMC	human peripheral blood mononuclear cells
IPTG	isopropyl-ß-D-thiogalaktopyranoside
LC/EI-MS	Liquid chromatography/Electrospray Ionization-Mass spectroscopy
OD	Optical density
ORN	oligoribonucleotide
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PMA	phorbol-12-myristate-13-acetate
RRM	RNA recognition motif
rt	room temperature
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
RT-PCR	reverse transcription polymerase chain reaction

SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
ss	single stranded
TEAAc	triethylammonium acetate
TMR	carboxytetramethylrhodamine
UTR	untranslated regions

EXAMPLES:

EXAMPLE A – Experimental protocols:

a) Preparation of fluorescently labeled RNA. 5' amino-C6 modified RNA is synthesized on an 394A synthesizer (Applied Biosystems) using 5'-O-dimethoxytrityl-2'O-triisopropyloxymethyl- protected β'-cyanoethyl-(*N,N*-diisopropyl-)nucleotide phosphoramidites (Glen Research) adopting published procedures (see e.g. Chaix C. et al., Nucleic Acids Symp. Ser. 1989, (21):45-6; Scaringe S.A. et al., Nucleic Acids Res. 1990, 18 (18):5433-41) and manufacturer's protocols. The ORNs are cleaved from the support, base-, phosphate- and 2'-deprotected and purified by denaturing polyacrylamide gel electrophoresis following standard protocols. RNA concentrations are calculated from UV-absorption at 260 nm according to the Bouguer-Lambert-Beer Law, using the exact molar extinction coefficient at 260 nm as determined according to reference (Gray D.M. et al., Methods in Enzymology 1995, 246:19-34). All ORNs are > 99 % pure according to analytical RP-HPLC analysis (VYDAC C_{18} column, 5µm, 300 Å, 4.6 mm x 250 mm, in TEAAc (0.1 M, pH 7.0) with gradient elution, 0 - 50 % CH_3CN in 45 min, UV-detection at 260 nm). TMR (Molecular Probes) is attached to the 5' aminolinker in a standard reaction of the primary amine with a succinimidylester-activated fluorophore to form a stable carboxamide. Unreacted dye is hydrolyzed by addition of hydroxylamine-hydrochlorid. The labeled RNA is separated from the free dye by gel filtration, purified from unlabeled RNA by RP-HPLC and the concentration is determined by UV absorption spectroscopy as described above but with correction for the dye absorption at 260 nm.

3'UTRs are prepared by run-off transcription from dsDNA templates with T7 RNA polymerase (T7 MEGASCRIPT in vitro transcription kit, Ambion). The T7 promoter is incorporated into the transcription templates during PCR amplification, using primers encompassing the 3'UTRs of IL-2 and TNF-α (IL-2: nt 707-1035, TNF-α: nt 872-1568, GenBank accession numbers NM_000589 and NM_000594, respectively). The transcript is 3' terminally oxidized with Na(m-)IO_4 and coupled to hydrazide activated Cy3 (AP Biotech), essentially as described in reference (Qin P.Z. et al., Methods 1999, 18(1):60-70). The product is subsequently purified by RP-HPLC as described for synthetic ORNs, desalted and transferred into aqueous solution by gel filtration. A 1:1 labeling stoichiometry is controlled by determination of the Cy3 and RNA concentration by UV/VIS absorption spectroscopy with correction for the dye absorbance at 260 nm.

Preparation of recombinant human HuR. The coding sequence for full-length HuR (amino acids 1-326, RefSeq accession: NP_001410) is amplified from cDNA prepared from activated human T-lymphocytes. The product is cloned directionally into the NdeI and SapI sites of the vector pTXB1 (IMPACT™-CN system, New England Biolabs), allowing C-terminal fusion with an intein-chitin binding domain tag without additional amino acid insertion. The fusion protein is expressed in E.coli ER2566 (New England Biolabs) upon induction with IPTG (1mM, for 6 hours at 28°). The bacterial cells are lysed by successive freezing/thawing cycles in a buffer of Tris/Cl (tris(hydroxymethyl)aminomethane, 20 mM pH 8.0), NaCl (800 mM), EDTA (1 mM) and Pluronic F-127 (0.2 % w/v, Molecular Probes). After DNA digestion, the lysates are cleared by ultracentrifugation and the fusion protein is captured onto chitin agarose beads (New England Biolabs). After extensive washing with lysis buffer, the recombinant protein is recovered by thiol-induced on-column self-splicing of the intein tag with 2-mercaptoethanesulfonic acid (sodium salt, 50 mM) for 12 hours at 4° (see e.g. Cantor E.J. et al., Protein Expr. Purif. 2001, 22(1):135-40). Any co-eluted intein tag and uncleaved fusion protein are removed from the eluate in a second, subtractive affinity step. The protein is transferred into the storage buffer (Na_2HPO_4/NaH_2PO_4 (25 mM) pH 7.2, NaCl (800 mM), Pluronic F-127 (0.2 % w/v)) by gel filtration (DG-10 columns, Bio-Rad), shock-frozen in small aliquots in liquid nitrogen and stored at –80°. Under these conditions, full length HuR is soluble without presence of higher aggregation states (analytical size exclusion chromatography), and shows the characteristic CD-spectra for RRM domains (Manival X. et al., Nucleic Acids Res. 2001, 29(11):2223-30). The protein is > 99 % pure according to LC/EI-MS, RP-HPLC and SDS-PAGE analysis. N-terminal sequencing reveals a correct N-terminus quantitatively missing Met_1. For a precise determination of the concentration, purified HuR is lyophilized, dissolved in guanidinium hydrochloride (6 M) and the concentration is determined by UV-spectroscopy according to reference (see Gill S.C. et al., Anal. Biochem. 1989, 182(2):319-26). This solution is used as external standard for determination of HuR concentrations by RP-HPLC quantification.

2D-FIDA-anisotropy HuR-RNA binding assay. The fluorescently labeled RNA is thermally denatured for 2 minutes at 80° in assay buffer (PBS, Pluronic-F-127 (0.1 % w/v), $MgCl_2$ (5 mM)), refolded by cooling to rt (-0.13 °C s^{-1}) and diluted to 0.5 nM, which ensures an average of < 1 fluorescent particles in the confocal volume in the described setup (Ecotec BA, 2001, 2D-FIDA Quick Guide, Hamburg). The accurate concentration in each sample is determined based on the particle number derived from a parallel FCS evaluation and the size of the confocal volume, as given by the adjustment parameters for the point spread

function (EVOTEC BioSystems, 2001). Fluorescently labeled RNA is titrated against increasing concentrations of recombinant HuR (at least 11 titration points). HuR-RNA samples are incubated for at least 15 minutes at rt prior to each measurement.

HuR-RNA complex formation is monitored under true equilibrium conditions by determination of the fluorescence anisotropy with 2D-FIDA. Measurements are performed in 96 well glass bottom microtiter plates (Whatman) on an EvotecOAI PickoScreen instrument at ambient temperature (constant at 23.5°). The Olympus inverted microscope IX70 based instrument is equipped with two fluorescence detectors, a polarization beamsplitter in the fluorescence emission path and an additional linear polarization filter in the excitation path. A HeNe laser (λ = 543 nm, laser power = 495 µW) is used for fluorescence excitation. The excitation laser light is blocked from the optical detection path by an interference barrier filter with OD = 5. A 0.5 nM solution of TMR in assay buffer is used for the adjustment of the confocal pinhole (70 µm) and for the determination of the G-factor of the instrument (Lakowitcz J.R. Principles of Fluorescence Spectroscopy, 2 ed., New York, 1999, Plenum Publsihers). 10 FIDA measurements are performed for each well at a measurement time of 10 seconds and a dwell time of 40 µseconds. The molecular brightness q is extracted from the 2D-FIDA raw data for each polarization channel using the FIDA algorithm (Kask P. et al., Biophys. J. 2002, 78(4):1703-13). Fitting parameters: background intensity in both channels as determined in a separate measurement of the buffer (usually ≈ 0.5 kHz), confocal volume parameters A0 and A1 as determined in the adjustment measurement with TMR (usually A0 ≈ -0.4 and A1 ≈ 0.08), single component fit. The anisotropy r is calculated from the molecular brightness for parallel and perpendicular polarization by equation [V] (Lakowicz, 1999, see above) and averaged from the 10 consecutive measurements.

$r = \frac{q_{II} - G \cdot q_{\perp}}{q_{II} + 2 \cdot G \cdot q_{\perp}}$	[V]

where q_{II}, $q_{\perp}$ are the: molecular brightnesses in parallel and perpendicular polarization channels and G denotes the G-factor of the instrument.

The anisotropy data are fitted based on the exact algebraic solution of the binding equation describing the average steady-state anisotropy signal r in dependence of the degree of 1:1 complex formation derived from the law of mass action (Daly T.J. et al., J.Mol.Biol. 1995, 253(2):243-58) to extract the equilibrium dissociation constant K_d^{app} (nonlinear least square regression, GraFit 5.0.3, Erithacus software, London):

$$r = r_{min} + \frac{(r_{max} * \frac{1}{Q} - r_{min}) * \left[([RNA_0] + [HuR_0] + K_d^{app}) - \sqrt{([RNA_0] + [HuR_0] + K_d^{app})^2 - 4 * [RNA_0] * [HuR_0]}\right]}{2 * [RNA_0]} \quad [VI]$$

where $[RNA_0]$: total concentration of RNA, $[HuR_0]$: total concentration of HuR, r_{min}: anisotropy of free RNA, r_{max}: anisotropy of RNA-HuR complex, r: average anisotropy for the steady-state equilibrium at the given HuR_0 and RNA_0 concentrations; Q: quenching, for 2D-FIDA-anisotropy measurements, $Q = q_{tot(max)} / q_{tot(min)}$ at $q_{tot} = q_{II} + 2\,q_{\perp}$; q_{II} . All presented data are averages from at least three independent experiments.

1D-FIDA HuR mRNA binding assay. The labeled mRNA or 3'UTR is thermally denatured for 2 minutes at 80° in assay buffer (PBS, Pluronic-F-127 (0.1 % w/v), $MgCl_2$ (5 mM)) and refolded by cooling to rt (-0.13 °C s^{-1}). Opener, closer or negative control ORNs (MWG Biotech, sequences see Table 1) are added to final concentrations between 0.5 and 100 nM. The final concentration of Cy3-labeled mRNA is 0.5 nM, accurate particle numbers are determined as described for the 2D-FIDA anisotropy measurements.

The labeled mRNA is titrated against increasing concentrations of HuR in presence and absence of openers or negative control ORNs. HuR-mRNA complex formation is monitored with 1D-FIDA by a determination of the molecular brightness change of Cy3 induced by HuR binding to the RNA. A HeNe laser (λ = 543 nm, laser power = 495 µW) is used for fluorescence excitation, the optical setup is analogous to the setup for 2D-FIDA anisotropy measurements, using one detection channel only and no polarization beam splitters in the optical paths. The molecular brightness q is extracted from the 1D-FIDA raw data using the FIDA algorithm and averaged from 20 consecutive measurements (10 seconds each). The molecular brightness data are fitted based on an equation analogous to equation [VI], adapted for fluorescence intensity measurements:

$$q = q_{min} + \frac{(q_{max} - q_{min}) * \left[([RNA_0] + [HuR_0] + K_d^{app}) - \sqrt{([RNA_0] + [HuR_0] + K_d^{app})^2 - 4 * [RNA_0] * [HuR_0]}\right]}{2 * [RNA_0]} \quad [VII]$$

q_{min}: molecular brightness of free RNA, q_{max}: molecular brightness of RNA HuR complex, q: average molecular brightness for the steady-state equilibrium at the given HuR_0 and RNA_0 concentrations. All presented data are averages from at least three independent experiments.

Preparation and stimulation of cells. hPBMC are isolated from heparinized blood by Ficoll-Hypaque centrifugation, washed with PBS containing BSA (15 % w/v), resuspended at 2 x 10^6 ml^{-1} in RPMI 1640 (Gibco/BRL) supplemented with heat-inactivated FCS (10% v/v), L-glutamine (2mM), streptomycin (100 µg ml^{-1}) and penicillin (100u ml^{-1}) and incubated in a 37° CO_2 incubator. hPBMC are stimulated for 4 hours with PMA (25 ng ml^{-1}, Sigma-Aldrich) and anti-CD3 mAb (1µg ml^{-1}, Pharmingen).

Co-immuneprecipitation of HuR-mRNA complexes. For each immuneprecipitation, 5 x 10^6 nonstimulated cells are washed with PBS/BSA and lysed at 4 °C in a hypotonic buffer (100 µl, Tris/Cl (10 mM) pH 7.5, NaCl (10 mM), EDTA (10 mM), Protease Inhibitor (Complete Mini EDTA free Protease Inhibitor Cocktail, Roche; 3 tablets per 50 ml lysis buffer) and Nonidet-P-40 (0.5 % v/v)). RNAsin (0.4 u ml^{-1}, Promega) and SuperasIn (0.2 u ml-1, Ambion) are added to inhibit unspecific RNA degradation. The lysates are centrifuged for 4 minutes at 15,000 x g and 4° to pellet nuclei. The cleared lysates are incubated for 5 minutes with anti-HuR mAb (5 µg ml^{-1}, 19F12, Molecular Probes) at 4° in presence and absence of opener or negative control ORNs (at 2.5 or 10 µM). After addition of biotinylated anti(mouse) IgG mAb (10 µg ml-1, Amersham Pharmacia), the immunecomplexes are captured on streptavidin sepharose beads (Amersham Pharmacia). The beads are washed thoroughly with lysis buffer. HuR and the complexed mRNA are eluted under acidic conditions (Glycin/HCl (50 mM, pH 2.5), NaCl (50 mM), prewarmed to 95 °C). The eluates are passed by centrifugation through BioSpin gel filtration columns (BioRad), pre-equilibrated with H_2O. Co-precipitated RNA is quantified by real-time RT-PCR.

mRNA decay. 5 x 10^6 stimulated hPBMC are lysed in lysis buffer (250 µl) as described above, in presence or absence of opener, closer or negative control ORNs at 10, 25 or 40 µM. mRNA degradation is initiated in the cleared lysates by addition of $MgCl_2$ (net concentration of 5 mM free Mg^{2+}). The degradation reaction is proceeded at rt and stopped after various timepoints between 2 and 70 minutes incubation (50 µl aliquots for each timepoint) by addition of EDTA and guanidinium isothiocyanat containing buffer (Qiagen). RNA is isolated using the RNeasy Miniprep RNA isolation kit (Qiagen) according to the manufacturers protocol, with DNAse I treatment for elimination of residual DNA.

Quantitative real-time RT PCR. RNA is reverse transcribed to cDNA using the TaqMan RT PCR reagents (Applied Biosystems) and random hexamers for priming following standard protocols. Control reactions for genomic DNA contamination are performed without addition of reverse transcriptase. Quantitative RT-PCR is performed with SYBR Green detection on an ABI7700 instrument (Applied Biosystems). EF-1α is used as endogenous control. The

primers used for RT-PCR are specified in Table 2. The ΔΔCt method is used for relative quantification of mRNA levels (as described eg. in (Applied Biosystems 2000) using in vitro transcribed IL-2 mRNA for calibration. All presented data are averages from at least 5 identical independent samples and representative of at least two independent experiments using cells from independent donors.

EXAMPLE B: Computational Protocols

Calculating the accessibility $p_\bullet^M$ of an RNA using constrained partition functions. We describe here the calculation of $p_\bullet^M$ for secondary structure elements defined by a sequence pattern with a secondary structure constraint, e.g. NNUUNNUUU in fully single stranded conformation. $p_\bullet^M$ can be calculated directly as set out for $p_\bullet$ in equation [III]. To avoid numerical errors, $p(a)$ is not calculated as a fraction of partition functions, but as a difference of ensemble free energies, $p(a) = \exp((W - W_a)/RT)$, where W is the ensemble free energy of the RNA sequence and W_a is the ensemble free energy of the thermodynamic secondary structure ensemble constrained to the structures where all sites in a form the particular secondary structure element. Ensemble free energies may be calculated using standard RNA secondary structure prediction software (Zuker M. Curr.Opin.Struct.Biol. 2000, 10(3):303-10). We have used the RNA library distributed with the Vienna RNA Package (Hofacker I.L. et al. Monatshefte Chemie 1994, 125:167-88), which is based on free energy parameters described in Mathews D.H. et al., J.Mol.Biol.1999, 288(5):911-40.

Calculating the accessibility $p_\bullet^M$ of an RNA using secondary structure samples. Alternatively, $p_\bullet^M$ can be approximated by counting the occurrence $n_\bullet$ of accessible structures in a set of (suboptimal) RNA secondary structures of size n, with thermodynamic equilibrium distribution. Such sets of RNA secondary structures can be generated using stochastic backtracking (Tacker M. et al., Eur.Biophys.J. 1996, 25:115-30; Ding Y. et al. Nucelic Acids Res. 2003, 31(24):7280-301), which is implemented in *RNAsubopt*, a program distributed with the Vienna RNA Package (Hofacker I.L. 1994, see above). The accessibility is then approximated as $p_\bullet^M \approx n_\bullet / n$. The approximate $n_\bullet / n$ converges to the true $p_\bullet^M$ with increasing n.

Calculating the accessibility $p_\bullet^{MO}$ for an oligonucleotide-RNA hybrid using partition functions. The thermodynamics of RNA-RNA hybridization is well understood (Dimitrov R.A.

et al. Biophys.J. 2004, 87(1):215-26). However, there is currently no implementation of an RNA secondary structure prediction which considers all possible structures of hybrids and is suitable to study the impact of hybridization on the accessibility directly. We therefore assume that all secondary structures of the RNA-oligonucleotide duplex can be approximated by those secondary structures of the RNA where the target site of the oligonucleotide cannot form internal base pairs, i.e. the RNA nucleotides which participate in the hybridization are constrained to be single stranded. Consequently, $p_{\bullet}^{MO}$ can be calculated like $p_{\bullet}^{M}$ using equation [III], but with an additional constraint t for the single stranded nucleotides due to hybridization:

$p_{\bullet}^{MO}(a) = Z(a \cup t) / Z(t)$	[VIII]

where $(a \cup t)$ means that both constraints need to be fulfilled simultaneously. Again, the calculation is performed using ensemble free energies to minimize numerical errors as detailed for the calculation of $p_{\bullet}^{M}$.

Calculating the accessibility $p_{\bullet}^{MO}$ of an RNA using secondary structure samples.

Analogous to the calculation of $p_{\bullet}^{M}$, $p_{\bullet}^{MO}$ can be approximated using secondary structure samples. It is again assumed that the effect of the hybridization is limited to deterring the hybridized nucleotides in the RNA from internal base pairing. The frequency of accessible structures $n_{\bullet}(t)$ is counted in a set of secondary structures which are constrained to be single stranded in the region of hybridization. In analogy to the notation used for the partition function approach, the accessibility is approximated as $p_{\bullet}^{MO} \approx n_{\bullet}(t) / n(t)$, where $n(t)$ is the total number of secondary structures in the constrained set.

Example 1: Manipulating the association of HuR to the IL-2 mRNA and thereby the IL-2 mRNA stability

Openers, closers and negative control ORNs are constructed for the IL-2 and TNF-α mRNAs and experimentally validated *in vitro* and in human primary cell lysates.

The plot showing the effect of the hybridization of 20mer ORNs on the fraction of accessible RNA structures computed for the design of opener and negative control ORNs for IL-2 is given in Figure 2. Remarkably, a significant accessibility increase is restricted to a limited number of "hotspots" within the mRNA, mainly located outside but in proximity to the HuR binding sites. At most other positions, hybridization leaves the local ARE conformation.

mostly unaffected. From these hotspots, 4 potential opener ORN as well as 2 negative control ORNs are selected for experimental characterization (Table 1). In a first step, we use ss ORNs.

The opener effect is initially validated *in vitro*. As measured in a 1D-FIDA assay, HuR binds to the IL-2 3'UTR (281 nt) with significantly higher affinity in presence of any of the openers (Figure 4A). The affinity increase induced by the opener correlates with the opener concentration (Figures 4B and C). Additionally, for Op1 the effect reverted at concentrations beyond a certain threshold (1.6 nM with 2.5 nM IL-2 3'UTR) and the HuR IL-2 3'UTR affinity is again reduced (Figure 4C). One possible explanation might be that, above certain concentrations, this particular sequence hybridizes also to secondary sites within the IL-2 3'UTR and thereby induces an adverse conformational rearrangement.

Negative controls are performed with two IL-2 3'UTR specific 20mers which do not affect the accessibility p(ssNNUUNNUUU). As demonstrated in Figure 4, both ORNs do not influence the HuR – IL-2 3'UTR association.

To verify that the opener ORNs also function in a more complex cellular environment, we quantify endogenous HuR–IL-2 mRNA association in a cellular system. Cytoplasmic lysates of hPBMC are treated with the opener ORNs. This experimental approach allows to exclude transcriptional effects of the openers. Defined opener concentrations are achieved and cellular stress responses induced by opener transfection are excluded from the experiment. IL-2 mRNA-HuR complexes are co-immunoprecipitated in presence or absence of the opener and HuR-bound IL-2 mRNA is quantified by real-time RT-PCR. Unspecific immunoprecipitation is excluded using a control antibody (goat IgG, data not shown). Both openers increase the level of HuR-IL-2 mRNA association up to 6.5 fold in a concentration dependent manner (Figure 5). No increase in HuR bound IL-2 mRNA is observed with the negative control ORN N_2 or with the TNF-α specific opener Op_T at the same conditions.

To validate the potential of the openers to modulate mRNA levels we test whether the induced increase in HuR complex formation would also antagonize the rapid ARE dependent mRNA degradation. We monitor IL-2 mRNA decay - which we observed to be Mg^{2+} dependent - in hPBMC lysates in the presence and absence of either openers Op_1, Op_2 as well as N_2. Upon Mg^{2+} addition the amount of remaining IL-2 mRNA is quantified over time by real-time RT-PCR. In absence of any opener, endogenous IL-2 mRNA is rapidly degraded ($t_{1/2}$ = 8.34 ± 0.96 minutes), while the mRNA of a non ARE gene (EF-1α) is stable throughout the observation time of 70 minutes (Figure 6). The observed halflives are comparable to previously described values (e.g. Raghavan et al., Nucleic Acids Res. 2002,

30(24):5529-38; $t_{1/2}$ (IL-2 mRNA) = 17 ± 10 minutes in human primary T-cells), indicating that such a degradation system is a valid approximation of an *in vivo* situation. In presence of opener Op_1 (c=10 µM), this degradation is completely arrested over a period of 15 minutes (Figure 6A). At that time untreated IL-2 mRNA is already degraded by 79.9 %. Also at prolonged incubation, the decay is significantly slowed down. At 40 µM concentration (Figure 6C), Op_1 blocks the degradation over the entire incubation time of 70 minutes. Op_2, which targets another HuR binding site, shows a similar stabilizing effect (Figure 6, Op_1 and Op_2 at concentrations of 25 µM (B) and 40 µM concentration (C)). The degradation kinetics of the IL-2 mRNA remains unaffected by hybridization with the negative control ORN N_2 (N_2 at 10 µM; $t_{1/2}$ = 6.82 ± 1.96 minutes). To ensure that the opener induced IL-2 mRNA stabilization is indeed a specific effect, we monitor mRNA stability of other ARE containing HuR targets in presence or absence of our IL-2 specific openers. Neither TNF-α nor IL-1β mRNA decay are influenced by the IL-2 specific openers (Figure 7A and B).

Example 2: Manipulating the association of HuR to the TNF-α mRNA and thereby the TNF-α mRNA stability.

The plot showing the effect of the hybridization of 20mer ORNs on the fraction of accessible RNA structures computed for the design of opener and negative control ORNs for TNF-α is given in Figure 8. Again, significant accessibility changes are clustered into "hotspots" in the mRNA, located proximal but also distant to the HuR binding sites. From these hotspots, one potential opener ORN as well as a putative closer and a negative control ORN are selected for experimental characterization (Table 1). In a first step, we use ss ORNs.

The opener effect is validated *in vitro*. As measured in a 1D-FIDA assay, the closer Cl_T significantly decreased the HuR affinity to the TNF-α 3'UTR (697 nt). The effect correlates with the closer concentration and approaches a maximum effect at the highest closer concentration (5 nM), with an affinity decrease by ~ 2.5 fold (Figure 9A) Conversely, the affinity is increased by the opener Op_T by a factor of up to ~ 2 (for Op_T at 0.25 nM, Figure 9B). Both effects are quantitatively consistent with the predicted accessibility change (Figure 8). At higher concentrations of Op_T, the effect however revertes and the HuR-TNF-α 3'UTR affinity is again reduced. One possible explanation might be that, above certain concentrations, this particular sequence hybridizes also to secondary sites within the TNF-α 3'UTR and thereby induces an adverse conformational rearrangement. Importantly, hybridization with the negative control oligo N_T does not change the HuR-TNF-α 3'UTR affinity (Figure 9C).

Finally, we test whether the induced increase in HuR complex formation would also antagonize the rapid ARE dependent mRNA degradation. We monitor TNF-α mRNA decay in hPBMC lysates in the presence and absence of Op_T. Upon Mg^{2+} addition the amount of remaining TNF-α mRNA is quantified over time by real-time RT-PCR. As a control, we measure the effect of Op_T on the decay of IL-1β as another ARE controlled cytokine mRNA. In absence of Op_T, endogenous TNF-α mRNA is rapidly degraded ($t_{1/2}$ = 36.0 ± 2.2 min, Figure 10). The observed halflife is comparable to previously described values (e.g. Raghavan A. et al., Nucleic Acids Res. 2002, 30(24):5529-38), $t_{1/2}$ (TNF-α mRNA) = 25 ± 11 minutes in human primary T-cells), indicating that such a degradation system is a valid approximation of an *in vivo* situation. In presence of Op_T at 25 µM, the degradation is arrested over an incubation time of 40 minutes. At that time, the degradation of untreated TNF-α mRNA has already proceeded to 53.1 %. Also at prolonged incubation, the decay is significantly slowed down. Importantly, the mRNA decay of IL-1β mRNA as another ARE containing HuR target is not influenced by Op_T, which demonstrates that the opener induced TNF-α mRNA stabilization is indeed a specific effect.

Table 1

Target mRNA	RefSeq	Opener/ Closer	Control	Positions	Sequence (5'→3')
IL-2	NM_000586	Op_1		834-853	AATATAAAATTTAAATATTT
		Op_2		909-928	TAGAGCCCCTAGGGCTTACA
		Op_3		804-823	AAGGCCTGATATGTTTTAAG
		Op_4		920-939	TGAAACCATTTTAGAGCCCC
			N_1	757-775	AGTGGGAAGCACTTAATTAC
			N_2	950-969	CATAATAATAAATATTTTGG
TNFα	NM_000594	Op_T		1315-1334	ATCACAAGTGCAAACATAAA
		Cl_T		1269-1288	CTGGCTCCATGGGGAGGGCT
			N_T	1173-1192	TGAGGTCTTCTCAAGTCCTG
		Op_A		174-193	TCGGCCAGCTCCACGTCCCG
		Op_B		626-645	TCTGGTAGGAGACGGCGATG
		Cl_C		615-634	ACGGCGATGCGGCTGATGGT
		Op_E		1114-1133	ATTCCAGATGTCAGGGATCA
		Op_H		1340-1359	CATTCATCTGTAAATAAATA

Table 2

Target mRNA	GenBank Accession No	Primer	Sequence (5' → 3')
IL-2 mRNA	NM_000586	forward	TCACCAGGATGCTCACATTTAAGTT
		reverse	GGAGTTTGAGTTCTTCTTCTAGACACTGA
TNFα mRNA	NM_000594	forward	AGGCGGTGCTTGTTCCTC
		reverse	GTTCGAGAAGATGATCTGACTGCC
IL-1β mRNA	NM_000576	forward	gtacctgagctcgccagtga
		reverse	tcggagattcgtagctggatg
EF-1 alpha	NM_001402	forward	tttgagaccagcaagtactatgtgact
		reverse	tcagcctgagatgtccctgtaa